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14. ABSTRACT

This objective of this project is to optimize the nanoparticle delivery system for effective ocular delivery of siRNA in animal models in order to validate the therapeutic targets developed at USAMRICD. In collaboration with the Target Discovery Core and the *In Vivo* Target Validation Core led by Dr. Albert Ruff at USAMRICD, we have developed new method to control the size and shape of siRNA nanoparticles. More importantly, this method can compact nanoparticles to smaller size with higher stability in physiological media, optimized a protocol to surface-coat nucleic acid nanoparticles with hyaluronic acid and retained the stability of the nanoparticles, and identified the conditions using reversible crosslinking density to stabilize siRNA nanoparticles. In addition, we have demonstrated gene knockdown in several cell lines, and confirmed that disulfide reversible crosslinks are essential to successful transfection and gene knockdown. In a pilot study with Dr. Ruff, we have identified feasible injection parameters and analysis protocols, although initial testing did not show positive gene knockdown with PEGylated spherical nanoparticles following sub-conjunctival injection, we plan to continue test the newer generation of rod and worm-like nanoparticles, which have shown promising results in other animal models.

15. SUBJECT TERMS nothing listed

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1. INTRODUCTION:

This proposed study is to optimize the nanoparticle delivery system for effective ocular delivery of siRNA in animal models in order to validate the therapeutic targets developed at USAMRICD. In collaboration with the Target Discovery Core and the *In Vivo* Target Validation Core led by Dr. Albert Ruff at USAMRICD, the team plans to develop potential therapeutic targets for the development of treatments to alleviate the effects of vesicant exposure for the warfighter. The specific tasks include: (1) To optimize nanoparticle tissue retention and cell uptake by conjugating cell adhesion ligand to nanoparticles and by surface coating of hyaluronic acid to improve the mucoadhesive property of the nanoparticles; extend siRNA knockdown duration by tuning the density of reversible crosslinks in nanoparticle core; and confirm that these modifications will not affect the nanoparticle properties; (2) To correlate optimization parameters of the nanoparticles (prepared in Task 1) with the gene knockdown efficiency following ocular delivery; and compare the efficiencies of topical application and subconjunctival injection of the nanoparticles. We had planned to complete the subconjunctival administration of the nanoparticles in Dr. Ruff's Lab at USAMRICD. (3) To confirm and optimize knockdown of therapeutic targets *in vivo*; characterize the expression kinetics; and identify cell types that are transfected by nanoparticles in collaboration with Dr. Ruff.

2. KEYWORDS:

Nanoparticles, non-viral vector, siRNA, gene knockdown, ocular delivery

3. OVERALL PROJECT SUMMARY:

Over the past year, we have developed an improved protocol to prepare a condensed siRNA nanoparticles with small and uniform size using PEGylated cationic carriers. Through optimization of the carrier structure, PEG density, and assembly conditions, we have prepared stable siRNA nanoparticles with discrete and compact size of 40 to 60 nm (number average). We have tested two different cationic polymers, polyphosphoramidate (PPA) and linear polyethyleneimine (PEI) to confirm the applicability of this method to different carriers. In addition, we have tailored the method to effective condense siRNA into both spherical and rod-like particles. These nanoparticles showed much improved stability in buffer with physiological ionic strength. The smaller nanoparticles elicited much higher gene knockdown efficiency in rodent models following a site-specific injection and systematic delivery, suggesting the improved stability in physiological media in vivo. These results highlight the effectiveness of our strategy in condensing and stabilizing nanoparticles in promoting their gene knockdown activity *in vivo*. Using a similar protocol, we have also achieved effective condensation of siRNA/PEI-g-PEG nanoparticles to an average of 44 nm (Fig. 1). Through molecular dynamics simulation, we revealed the role of solvent quality and PEI–PEG hydrogen bonding in the assembly of lPEI-*g*-PEG/siRNA micelles. The micelle size was preserved after organic solvent removal by means of reversible disulfide crosslinking; we confirmed that the micelles maintained their size in water and physiological media. We have also confirmed size-dependent *in vivo* transfection efficiency following intravenous injection of the siRNA micelles in rats (Fig. 2). The gene knockdown efficiency in rat liver achieved by the smaller siRNA micelles was significantly higher than for the larger micelles prepared from the same copolymer carrier. The

condensation technique introduced here allows a simple and effective way to reduce siRNA particle size and provides a model platform for further study of the effect of particle size on *in vivo* cellular uptake, knockdown efficiency, biodistribution, and pharmacokinetics.

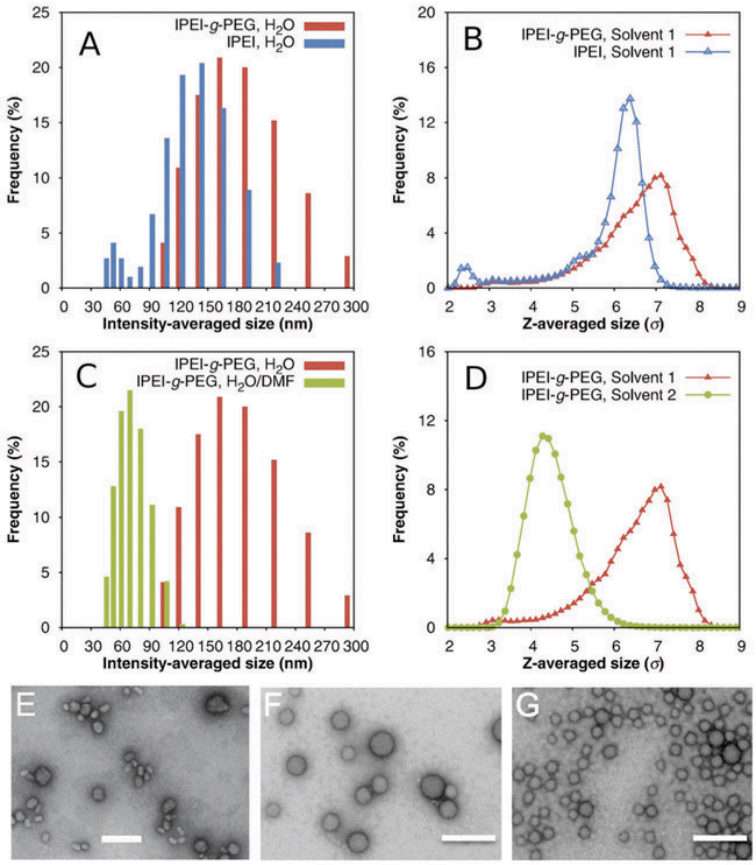

Fig. 1. Size distribution of lPEI-*g*-PEG/siRNA micelles in different solvents. (A) Size distribution of lPEI-*g*-PEG/siRNA micelles and lPEI/siRNA nanoparticles prepared in water, as determined by dynamic light scattering; (B) Simulation results for size distributions of lPEI-*g*-PEG/siRNA micelles and lPEI/siRNA nanoparticles in water; (C) Size distribution of lPEI-*g*-PEG/siRNA micelles prepared in pure water and 7:3 (v/v) DMF–water mixture; (D) Simulation results for size distribution of lPEI-*g*-PEG/siRNA micelles in pure water (labeled "Solvent 1") and 7:3 (v/v) DMF–water mixture (labeled "Solvent 2"); (E–G) TEM images of lPEI/siRNA nanoparticles (E), lPEI-*g*-PEG/siRNA micelles prepared in pure water (F), and lPEI-*g*-PEG/siRNA micelles prepared in DMF–water mixture (G), respectively. All scale bars represent 200 nm.

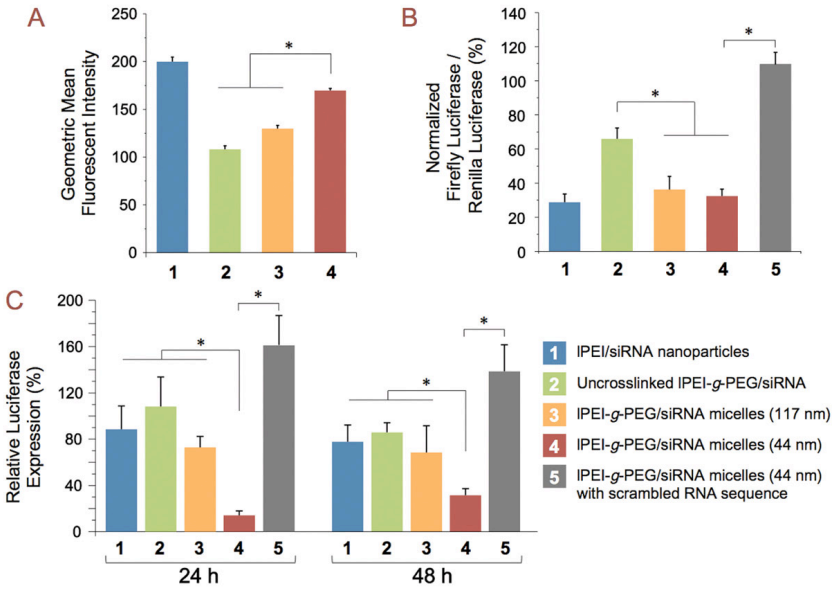

Fig. 2. Size-dependent transfection efficiency of lPEI-*g*-PEG/siRNA micelles. (A) *In vitro* cellular uptake of Alexa Fluor 488-labeled micelles in HepG2 cells. Bars represent mean ± SD (n = 3); (B) *In vitro* gene knockdown efficiency in HepG2 cells following 100 nM equivalent dose of siRNA. Bars represent mean ± SD (n = 3); (C) *In vivo* gene silencing in rat liver at 24 h and 48 h after administration of nanoparticles at a dose equivalent to 80 μg siRNA *via* tail vein injection. Bars indicate mean relative luciferase expression ± SD (n = 4–7). *p <0.05.

In addition, we have recently also developed conditions that allows for shape control over siRNA nanoparticles (Fig. 3). Using a reversible disulfide bond crosslinking method, we have shown these nanoparticles are stable in medium with physiological ionic strength. These nanoparticles also showed significant gene knockdown efficiency *in vitro* and *in vivo* in brain tissue. The results indicate that rod-shaped nanoparticles were most effective for the delivery of siRNAs to the CNS, highlighting the effect of nanoparticle shape on siRNA delivery *in vivo*.

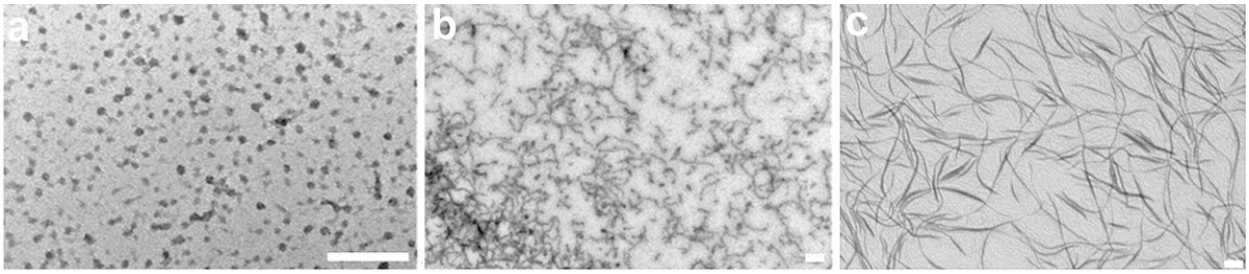

Fig. 3. Tuning the shape of siRNA nanoparticles ranging from spherical $LPEI_{17k}$-8%PEG_{10k}/siRNA nanoparticles (a), rod-like $LPEI_{17k}$-4%PEG_{10k}/siRNA nanoparticles (b), and worm-like $LPEI_{17k}$-2%PEG_{10k}/siRNA nanoparticles (c). Scale bars = 200 nm.

To improve the compatibility of the siRNA nanoparticles with ocular tissue, and improve the retention and uptake of these particles, we adopted a previously developed ionic crosslinking strategy to stabilize the lPEI/siRNA complex prior to HA coating through the incorporation of sodium triphosphate (Fig. 4a). This strategy allows for the formation of stable, discrete spherical particles with average diameters of 100 nm (Fig. 4b). Importantly, HA-coating also significantly improves the knockdown efficiency *in vitro* when tested in two different corneal cell lines, compared to particles without HA coating and without TPP crosslinking (Fig. 4c).

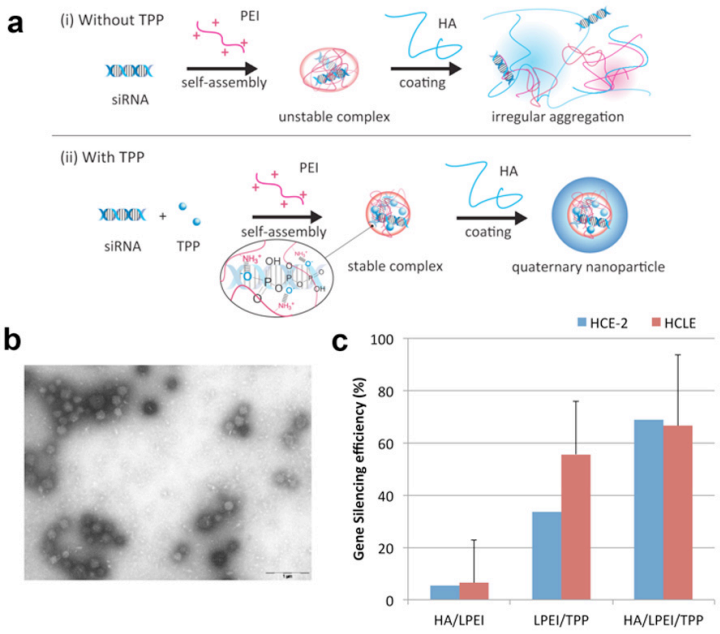

Fig. 4. (a) Preparation of lPEI/siRNA nanoparticles followed by HA-coating leads to irregular aggregation, unless TPP is used to stabilize the complex prior to HA-coating. (b) TEM image of HA-coated lPEI/TPP/siRNA nanoparticles; (c) Luciferase gene silencing efficiency mediated by siRNA nanoparticles in human corneal epithelial (HCE-2) and human corneal limbal epithelial (HCLE) cells with siRNA dose of 100 nM. Values are expressed as mean ± SEM (n=4).

4. KEY RESEARCH ACCOMPLISHMENTS:

- We have developed new method to control the size and shape of siRNA nanoparticles. More importantly, this method can compact nanoparticles to smaller size with higher stability in physiological media. Optimized a protocol to surface-coat nucleic acid nanoparticles with hyaluronic acid, and retain the stability of the nanoparticles. Identified the conditions using reversible crosslinking density to stabilize siRNA nanoparticles.

- We have demonstrated gene knockdown in several cell lines, and confirmed that disulfide reversible crosslinks are essential to successful transfection and gene knockdown.

- We have collaborated with Dr. Ruff and tested the first generation of nanoparticles (PEGylated spherical nanoparticles) following sub-conjunctival injection. However, this pilot study did not show positive gene knockdown with the initial nanoparticle formulation and dose. Nonetheless, we have identified the feasible injection parameters and analysis protocols. Part of the challenge is with the small injection volume, which required lyophilization and resuspension of nanoparticles in extremely high concentration (2 – 5 mg/ml). Severe aggregation was observed under this condition.

- Since the parent grant at USAMRICD was completed, we could not test new nanoparticles at Dr. Ruff's lab. Instead, we will focus the remaining work on gene knockdown efficiency of nanoparticles administered by topical application only at Johns Hopkins University.

5. CONCLUSION:

Vesicant injury has been unaddressed threat for the warfighters for decades. This proposed study is an important component of the VT-CCRP. The long-term goal is this program is to develop therapeutic strategies to effectively minimize injuries to ocular tissue resulting from vesicant exposure. This task of this project is to develop a nanoparticle system for delivery of the identified gene knockdown targets developed at USAMRICD. We have developed tailored siRNA nanoparticles packaging method to yield nanoparticles with controlled shape and size and improved stability. Over the extended few months, we will test the delivery efficiency of siRNA in eye tissue following topic delivery.

If successful, these nanoparticles will also find widespread application for treating ocular diseases. Subconjunctival injection can be easily achieved in humans; also various types of therapeutics have been effectively delivered via subconjunctival injection. It has also been shown that subconjunctival injection can effectively deliver nanoparticles, drugs or proteins to corneal mucosal cell types with low cytotoxicity. Furthermore, gene carriers developed here were selected with strong track records of safety and clinical testing. Thus, the gene knockdown approach developed here may be easily translated to future clinical treatments.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Manuscript in review:

- Wu J, Jiang X, Pan D, Crow MT, Mao HQ. Dual-sensitive Polymer/siRNA Nanoparticles with Enhanced Gene Knockdown Efficiency. Gene Therapy. (In Review)
- Qu W, Wu J, Mao HQ, Luijten E. Solvent induced size reduction of self-assembled siRNA/copolymer nanoparticles. Biomacromolecules. (In Review)
- Williford JM, Shyam R, Santos JL, Mao HQ. Methods and Applications of Nanoparticle Shape Control in Therapeutic Delivery. Biomaterials Science. (In review)

Invited review:

- Williford JM, Wu J, Ren Y, Archang MM, Leong KW, Mao HQ. Recent Advances in Nanoparticle-Mediated siRNA Delivery. Annual Reviews in Biomedical Engineering. 16 (7): 347-370 (2014). PMID: 24905873.

Conference Presentations:

- *Shyam R, Lee J, Ren R, Wong, P, Mao HQ. siRNA delivery mediated by shape-controlled nanoparticles. 2014 American Society of Gene and Cell Therapy. Podium Presentation.
- American Chemical Society National Meeting, Symposium on Biomacromolecules for Therapeutics and Diagnostic Delivery. Indianapolis, IN. September 8–12, 2013. (Invited)

7. INVENTIONS, PATENTS AND LICENSES:

- Shape-Controlled siRNA Nanoparticles for In Vivo Delivery of RNA Therapeutics. US Provisional Patent Application Serial No. 62/000,838 (Filed on 05/20/2014).

8. REPORTABLE OUTCOMES:

- A series of nanoparticles for siRNA packaging with small and compact size, and different shapes;
- A method for siRNA packaging and stabilization, and surface modification.

9. OTHER ACHIEVEMENTS:

Nothing to report.

10. REFERENCES:

None included.

11. APPENDICES:

None included.